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Antisense inhibition of cytosolic phosphorylase in potato plants (*Solanum tuberosum* L.) affects tuber sprouting and flower formation with only little impact on carbohydrate metabolism

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Summary

To determine the function of cytosolic phosphorylase (Pho2; EC 2.4.1.1), transgenic potato plants were created in which the expression of the enzyme was inhibited by introducing a chimeric gene containing part of the coding region for cytosolic phosphorylase linked in antisense orientation to the 35S CaMV promotor. As revealed by Northern blot analysis and native polyacrylamide gel electrophoresis, the expression of cytosolic phosphorylase was strongly inhibited in both leaves and tubers of the transgenic plants. The transgenic plants propagated from stem cuttings were morphologically indiscernible from the wild-type. However, sprouting of the transgenic potato tubers was significantly altered: compared with the wild-type, transgenic tubers produced 2.4 to 8.1 times more sprouts. When cultivated in the greenhouse, transgenic seed tubers produced two to three times more shoots than the wild-type. Inflorescences appeared earlier in the resulting plants. Many of the transgenic plants flowered two or three times successively. Transgenic plants derived from seed tubers formed 1.6 to 2.4 times as many tubers per plant as untransformed controls. The size and dry matter content of the individual tubers was not noticeably altered. Tuber yield was significantly higher in the transgenic plants. As revealed by carbohydrate determination of freshly harvested and stored tubers, starch and sucrose pools were not noticeably affected by the antisense inhibition of cytosolic phosphorylase; however, glucose and fructose levels were markedly reduced after prolonged storage. These results favour the view that cytosolic phosphorylase does not participate in starch degradation. The possible links between the reduced levels of cytosolic phosphorylase and the observed changes with respect to sprouting and flowering are discussed.

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Introduction

α-1,4-glucan phosphorylase catalyses the reversible release of glucose-1-phosphate from α-1,4-glucans. Compartment-specific phosphorylase isozymes from several plant species have been characterized with respect to their kinetic properties (Conrads *et al.*, 1986; Fukui *et al.*, 1987; Preiss *et al.*, 1980). The cytosolic isozyme, designated Pho2 (International Society for Plant Molecular Biology (ISPMB) number 263), exhibits a high affinity to large, highly branched polyglucans, such as glycogen (Shimomura *et al.*, 1982) or a high-molecular-weight heteroglycan (Yang and Steup, 1990). In contrast, the plastidic phosphorylase, Pho1 (ISPMB number 262) prefers maltodextrin and has a low affinity towards branched polyglucans, especially to glycogen (Steup, 1988).

In higher plants, several other α-glucan-metabolizing enzymes, such as amylases, debranching enzymes, D-enzyme and soluble glucan synthase; have been localized both inside and outside the chloroplast (Beck and Ziegler, 1989; Beers and Duke, 1988; Tacke *et al.*, 1991). Due to the compartmentation of the plant cell, transitory starch granules are only accessible to enzymes that reside inside the chloroplast, and therefore the extra-chloroplast enzymes are probably not immediately involved in the metabolism of transitory starch. The situation in the potato amyloplast with respect to the accessibility of storage starch to cytosolic enzymes is similar to that in chloroplasts at most developmental stages, but might be changed in tubers which are sprouting. The sprouting process is paralleled by starch degradation and a progressive disintegration of the compartmental integrity of the amyloplast (Isherwood, 1976; Kumar and Knowles, 1993a). Under these conditions, extra-plastidic degradative enzymes could have access to the starch grain and participate in starch mobilization (Ohad *et al.*, 1971). Another metabolic function for these non-chloroplast enzymes would be based on the existence of an oligo- or polysaccharide pool present in the same subcellular site. For the cytosolic phosphorylase, such an endogenous substrate might be represented by a high-molecular-weight heteroglycan, present in the cytosol, which strongly interacts with cytosolic phosphoryl-

ase (Steup *et al.*, 1991; Yang and Steup, 1990). At present it is not possible to discriminate between these alternatives, and the function of cytosolic phosphorylase remains unclear.

In order to investigate the role of the cytosolic isozyme of phosphorylase, the expression of the enzyme was inhibited in transgenic potato plants by expressing an antisense RNA. In the present study, the relevance of cytosolic phosphorylase for the growth and development of potato plants and tubers as well as its influence on starch turnover in both leaves and tubers and the effects on the soluble sugar content in tubers are analysed.

Results

Construction of a chimeric antisense cytosolic phosphorylase gene

To elucidate the physiological role of cytosolic phosphorylase in potato, transgenic plants with decreased activities of this isoform were regenerated. A PCR-amplified 2 kb long cDNA insert encoding potato cytosolic phosphorylase (Mori *et al.*, 1991) was inserted in antisense orientation between the constitutive 35S CaMV promotor (Franck *et al.*, 1980) and the polyadenylation signal of the octopine synthase gene (Gielen *et al.*, 1984) in the binary vector pBinAR (Höfgen and Willmitzer, 1990). The resulting construct, p35SαPho2 (Figure 1), was used to transform potato plants (*Solanum tuberosum* L. cv. Désirée) using *Agrobacterium*-mediated gene transfer (see Experimental procedures). Transgenic plants were selected in tissue culture on the basis of their kanamycin resistance.

Selection of plants with reduced levels of cytosolic phosphorylase

Sixty independent primary transformants were transferred to soil and grown under standardized greenhouse conditions (see Experimental procedures). The plants were analysed for Pho2 gene expression and phosphorylase activities in their leaves and tubers (Figure 2). The decreased accumulation of Pho2-specific transcripts was paralleled by a decrease in cytosolic phosphorylase activity. Seven primary transformants (c6, c7, c9, c14, c15, c16, c18) with strongly reduced levels of the isozyme in their tubers were selected for a more detailed analysis. When planted from stem cuttings, they were morphologically indistinguishable from wild-type potato plants (cv. Désirée), which served as controls. Unless stated otherwise, all further experiments were performed with plants grown from tubers.

Plants with reduced levels of cytosolic phosphorylase are not altered in carbohydrate metabolism during tuber development

In order to analyse the influence of inhibiting cytosolic phosphorylase in transgenic potato plants on carbohydrate metabolism, extracts from transgenic and wild-type plants were analysed for their starch content (both in leaves and tubers) and mono- and disaccharides such as glucose, fructose and sucrose (in tubers only). No change in the starch content was observed in fully expanded leaves from transgenic plants at the beginning as well as at the end of the light period (data not shown). The starch and soluble sugar (glucose, fructose and sucrose) content of freshly harvested tubers deficient in cytosolic phosphorylase was also not significantly altered compared with control plants (see Table 1). The specific gravity and the dry weight of freshly harvested tubers were determined, but no differences were observed (data not shown).

Starch has a complex granular structure and is composed of distinct amylopectin and amylose fractions (Kainuma, 1988), which suggests the existence of a highly ordered and precise pathway for its synthesis and degradation (Preiss, 1982). Even though the amounts of carbohydrates are unaffected, structural and physico-chemical changes in the starch from the trangenic potato plant could have occurred. Starch isolated from freshly harvested tubers of the transgenic and control plants was therefore analysed with respect to granule morphology, amylose and phosphate (P_i) content, chain length, paste behaviour and textural properties of gels formed by the respective pastes using standard starch analysis equipment. Neither the granule size nor the amount of amylose, as determined photometrically by the λ_{max} of the iodine–polysaccharide complex, and the amount of P_i bound at the C6 position of the glucose monomers, as measured in a coupled

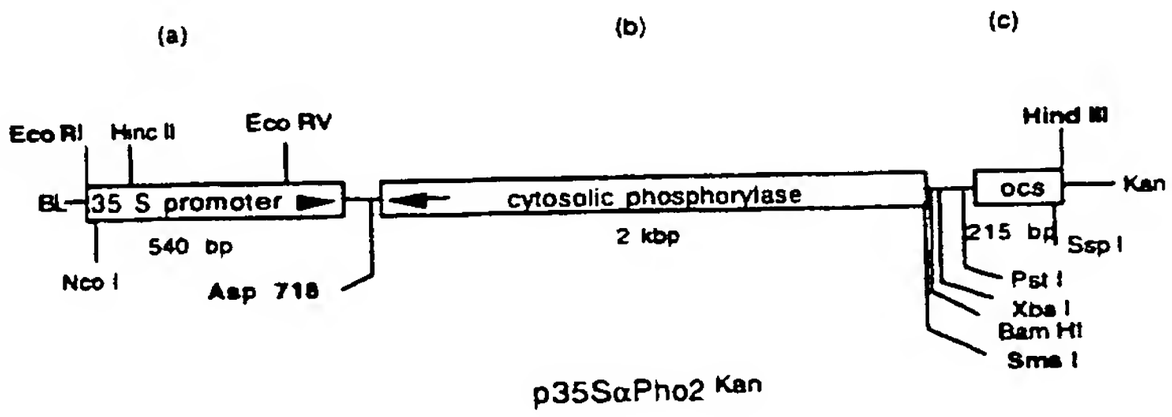

Figure 1. Structure of the chimeric cytosolic phosphorylase antisense gene, p35Sα-Pho2^{Kan}, used for the transformation of potato plants.
(a) A 540 bp fragment containing the 35S promotor of the CaMV; (b) a 2 kbp*Asp* 718/*Sma*I fragment containing part of cytosolic phosphorylase in antisense orientation; (c) the polyadenylation signal of the octopine synthase gene.

enzyme assay with glucose-6-phosphate dehydrogenase, were changed in starch isolated from tubers of the transformed plants (data not shown). Starch solutions were analysed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) after hydrolysis with isoamylase, α-amylase or β-amylase. No differences in the pattern or the distribution of the different products of enzymatic breakdown were detected between samples from transformed and untransformed plants after separation on CarboPac PA-100 columns (Dionex Co., Sunnyvale, CA, USA; data not shown). Both the paste properties of the starch samples (as tested by the Rapid Visco-Analyser) and the strength and adhesive force of starch gels (as tested by the QTS Texture-Analyser) revealed similar results for wild-type and the transgenic plants (data not shown). Taken together, these results indicate that no changes in the distribution and branching pattern of the glucans have occurred.

To elucidate possible changes in the amount and composition of malto- and other oligosaccharides, water- and ethanol-soluble sugars of freshly harvested tubers were analysed by HPAEC–PAD. The extracts were separated before and after degradation with amyloglucosidase on CarboPac PA-100 columns, but no changes could be detected between samples from transformed and untransformed plants (data not shown).

It was of interest to analyse whether the inhibition of cytosolic phosphorylase would lead to any changes with respect to the tuberization process of plants derived from tissue culture stem cuttings. The tuber number as well as the total tuber yield per transgenic plant were unaltered in comparison to wild-type plants, which was not the case when the plants were grown from seed tubers (see below).

These results demonstrate that, under the conditions tested, a reduction in cytosolic phosphorylase activity does not lead to detectable changes in carbohydrate metabolism in growing tubers, and hence to changes in tuber development.

Reduction of cytosolic phosphorylase in tubers of transgenic plants has no effect on starch mobilization, but influences tuber sprouting

Tubers represent the major storage sink organs of potato plants. Carbohydrates are stored in the tuber in the form

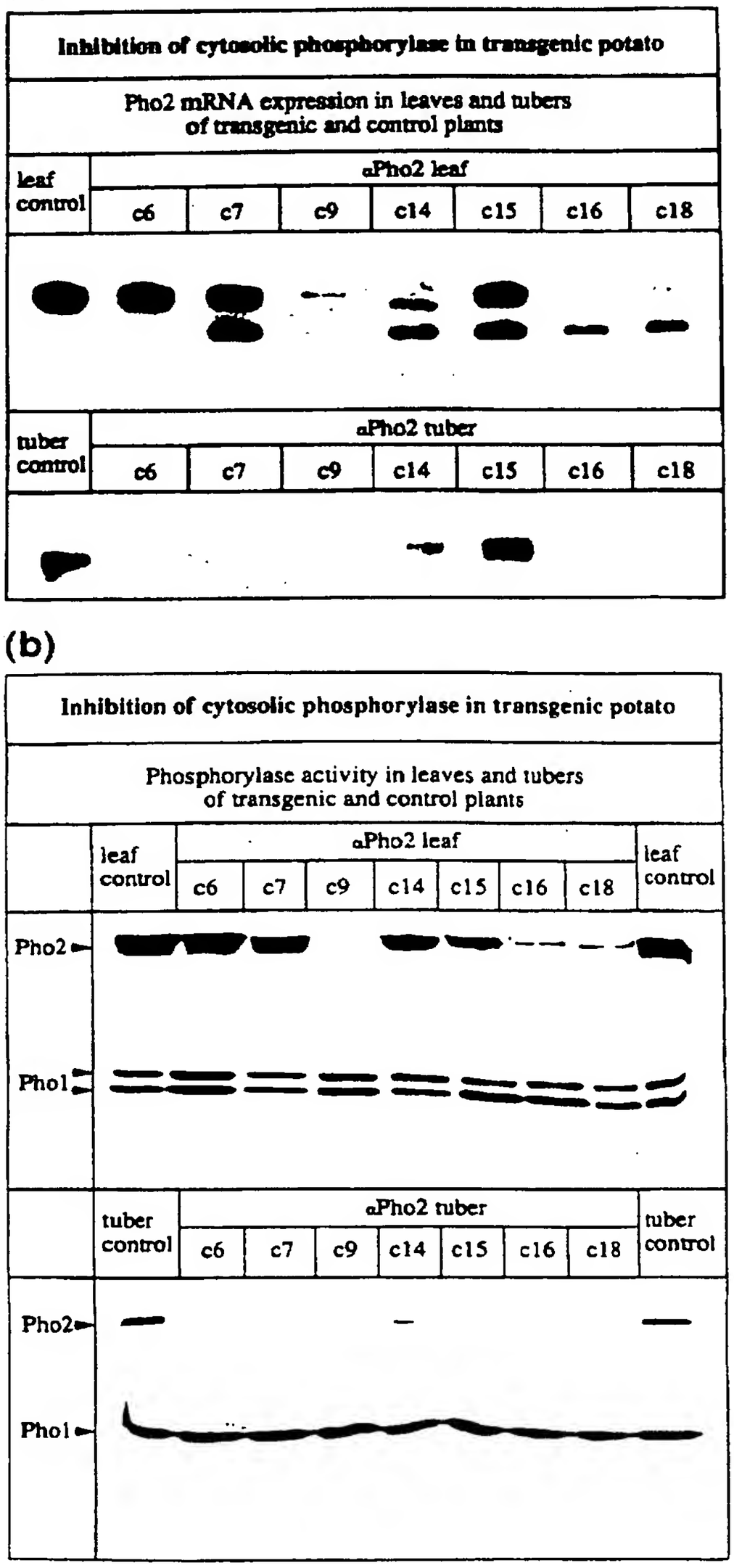

Figure 2. Analysis of leaves and tubers of transgenic potato plants inhibited for expression of cytosolic phosphorylase.
(a) Analysis of cytosolic phosphorylase mRNA expression in leaves and tubers of transgenic and control plants. Total RNA was separated on a denaturing agarose gel, blotted onto a nylon membrane and hybridized to the [^{32}P]-labelled cytosolic phosphorylase *Pst*I cDNA fragment of plasmid pSK–Pho2. The size of the transcript is approximately 2.6 kb. Below the sense RNA, the antisense RNA is detected in leaves of most of the transgenic lines. Equal amounts were loaded (20 μg/lane) for each tissue.
(b) Analysis of cytosolic phosphorylase activities in leaves and tubers of transgenic and control plants. Crude leaf and tuber extracts were resolved by non-denaturing polyacrylamide gel electrophoresis (discontinous system: separation gel containing 2.4% (w/v) glycogen). Aliquots of 20 μg leaf protein or 5 μg tuber protein were applied per lane. Electrophoresis was performed for 4 h at 100 V. The migration direction is from top (cathode) to bottom (anode). For activity staining, gels were incubated in a mixture containing 20 mM glucose-1-phosphate, and 100 mM citrate (pH 6.0) at room temperature overnight. The high-affinity Pho2 isozyme is strongly retarded by the immobilized polysaccharide, whereas the migration velocity of the low-affinity Pho1 isozyme is essentially unaffected.

of starch, which subsequently is mobilized during sprouting due to the action of starch-degrading enzymes (hydrolases and phosphorylases). Since cytosolic phosphorylase is localized in the cytosol, we determined whether cytosolic phosphorylase would have access to the starch granule in the process of sprouting due to membrane breakdown (Sowokinos *et al.*, 1987), and thereby influence starch mobilization and the formation of new sprouts. Tubers from plants containing the antisense gene were therefore stored at 20°C in the dark in parallel with tubers from control plants for different time periods. In order to exclude any effects which might be due to the history of the tubers, the material used was derived from plants that had been grown and harvested in parallel. Surprisingly, sprouting of cytosolic phosphorylase-deficient tubers was drastically enhanced in three independent experiments. The phenotype of these tubers is shown in Figure 3. In transgenic plants of the lines c6, c7, c9, c14, c15, c16 and c18, the tubers showed an increased number of growing buds and produced significantly more sprouts (2.4 to 8.1 times more sprouts after six months of storage at 20°C in the dark; Table 2). This phenotype was not observed in plants from the same transformation experiment, which did not show any or only a slight reduction of cytosolic phosphorylase activity (data not shown). In order to confirm that the reduction in cytosolic phosphorylase activity in the lines c6, c7, c9, c14, c15, c16 and c18 is also observed in the developmental stage of tuber sprouting, protein extracts derived from tubers which had been stored for seven months at 20°C in the dark were analysed for their cytosolic phosphorylase activity. An identical picture was seen as in growing tubers (data not shown).

However, the changes in sprouting behaviour do not coincide with any changes in the capacity for starch mobilization, as the starch content in tubers after different periods of storage was determined, but no significant differences between tubers from transgenic and control plants were observed (Table 1). The iodine staining of longitudinal sections of sprouting tubers both of wild-type and transgenic plants revealed that starch degradation occurs mainly in a region below the growing bud. The macroscopically determined staining pattern of tuber sections of the transgenic plants was more complex, simply because more buds sprouted than in wild-type tubers (data not shown).

HPAEC–PAD was again used to determine whether any

Table 1. Carbohydrate partitioning in tubers of transgenic potato plants with decreased levels of cytosolic phosphorylase

Plant	Experiment	Glucose	Fructose	Sucrose	Starch
Control	1[a]	5.72 ± 0.50	1.48 ± 0.09	14.13 ± 1.70	588.89 ± 32.39
	2[b]	4.09 ± 0.74	1.80 ± 0.34	15.44 ± 1.12	458.11 ± 31.25
	3[c]	2.19 ± 0.61	1.32 ± 0.31	14.39 ± 0.71	442.94 ± 33.45
	4[d]	8.78 ± 0.61	8.78 ± 1.08	43.58 ± 6.31	370.01 ± 22.43
	5[e]	12.21 ± 2.08	10.60 ± 1.70	80.08 ± 7.75	367.74 ±24.58
c9	1	5.76 ±0.81	1.21 ± 0.08	13.88 ± 0.67	553.49 ± 44.02
	2	3.30 ± 0.70	1.37 ± 0.21	16.57 ± 0.44	411.68 ± 24.13
	3	2.39 ± 0.66	0.89 ± 0.08	11.85 ± 0.75	430.34 ± 30.32
	4	5.07 ± 0.56	4.36 ± 0.52	42.36 ± 3.30	398.86 ± 23.78
	5	6.24 ± 0.91	4.13 ± 0.36	90.42 ± 6.18	343.20 ± 17.00
c16	1	4.58 ± 0.46	1.19 ± 0.17	12.35 ± 0.86	609.58 ± 31.40
	2	3.81 ± 0.65	1.06 ± 0.30	12.18 ± 0.95	405.86 ± 36.36
	3	2.27 ± 0.45	1.07 ± 0.27	13.89 ± 0.83	443.18 ± 26.60
	4	3.27 ± 0.28	3.62 ± 0.45	32.26 ± 3.88	343.68 ± 20.52
	5	3.59 ± 0.42	6.24 ± 0.74	72.85 ± 6.14	341.76 ± 17.00
c18	1	5.51 ± 0.64	1.35 ± 0.14	15.18 ± 1.16	598.04 ± 42.32
	2	3.50 ± 0.62	1.10 ± 0.23	17.06 ± 0.54	432.73 ± 29.61
	3	2.59 ± 0.75	1.24 ± 0.31	10.95 ± 0.45	435.98 ± 32.73
	4	5.76 ± 0.79	7.17 ± 1.22	38.15 ± 3.03	345.90 ± 35.48
	5	5.18 ± 0.80	4.83 ± 0.51	77.74 ± 4.30	347.67 ± 32.09

Values for glucose, fructose, sucrose and starch are given in µmol hexose per g fresh weight. Data were obtained from five independent plants clonally propagated from each individual transformant. Tubers were harvested at the end of the tuberization period (i.e. when the aerial parts of the plants were senescent) from plants grown under standardized greenhouse conditions, and stored for 1 to 13 months. Values are given as mean ± standard error of at least ten determinations from the core region of independent potato tubers of comparable fresh weight (8–16 g; n = 10–20).

[a]Freshly harvested tubers; [b]tubers stored for 4 weeks at 20°C in the dark; [c]tubers stored for 8 weeks at 20°C in the dark; [d]tubers stored for 7 months at 20°C in the dark; [e]tubers stored for 13 months at 20°C in the dark.

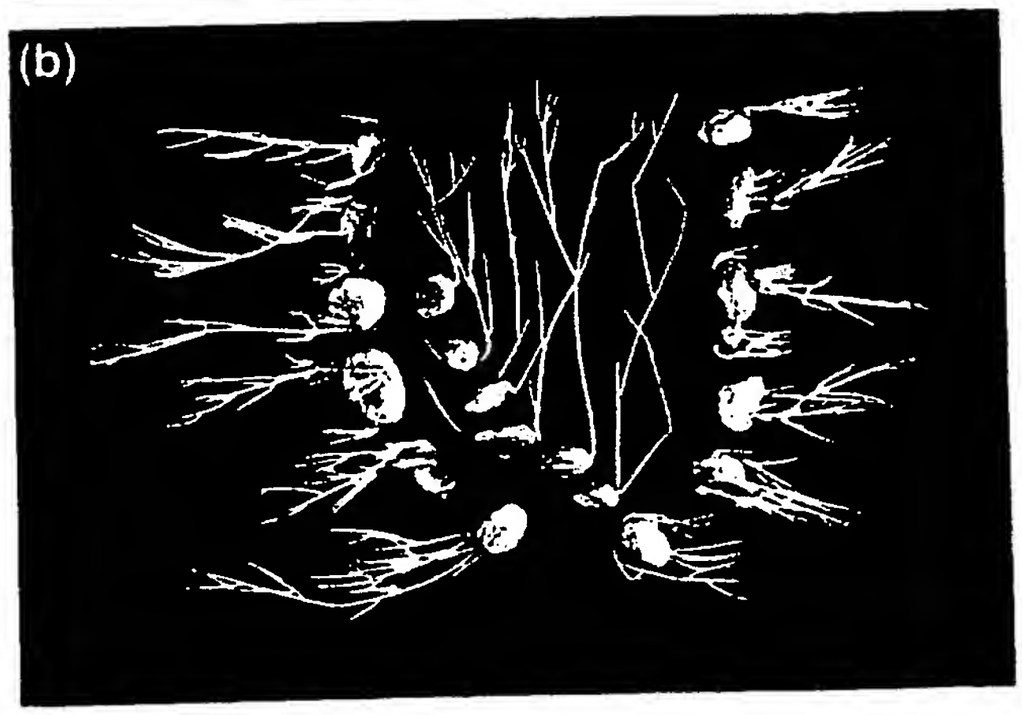

Figure 3. Inhibition of cytosolic phosphorylase activity in tubers leads to drastically altered sprouting behaviour.
(a) Sprouting tubers of transgenic potato plants (c9, left) and of control plants (right) after five months of storage at 20°C in the dark.
(b) Sprouting tubers of transgenic (c6, left; c7, right) and control plants (centre) after ten months of storage at 20°C in the dark. The effect of inhibited cytosolic phosphorylase activity on the number of sprouts formed by individual tubers as well as by single tuber buds is demonstrated.

changes in water or ethanol-soluble saccharides occurred after different periods of storage. As for growing tubers, no changes were observed on the level of malto- or other oligosaccharides, as the elution profile was indistinguishable between extracts derived from control and transgenic tubers after different storage periods. This was observed in the case of untreated extracts, as well as after hydrolysis with amyloglucosidase, which was used in order to distinguish between malto- and other oligosaccharides (data not shown).

However, significant changes were measured when glucose, fructose and sucrose levels were determined. A 40–60% decrease was observed in the transgenic tubers compared with wild-type controls in the case of glucose and fructose, whereas the sucrose levels remain unaltered. These changes are only detectable in tubers which have sprouted (after 7 and 13 months of storage), whereas no differences were measured in apparently dormant tubers (Table 1).

Alteration of tuber sprouting influences further plant development

The number of sprouts formed per seed tuber is critical for the yield produced by individual potato plants. Plants grown from seed tubers showed major differences both in the timing and intensity of stem growth (Figure 4). First, the emergence of sprout tips occurred one to three weeks earlier in transgenic tubers (data not shown). Second, two to three times more stems developed per transformed seed tuber (Table 3). Therefore we investigated whether or not the altered sprouting behaviour of tubers from the transgenic plants, leading to more sprouts per seed tuber, would result in changes in the tuber yield per plant. Unexpectedly, even the development of the aerial parts of the transgenic plants was altered. As evident from Figure 4, the transgenic plants showed an earlier onset of flowering, and often formed successive second or even third inflores-

Table 2. Influence of reduced cytosolic phosphorylase levels on tuber sprouting

Plant	Number of sprouts per tuber	Number of growing buds per tuber	Number of sprouts per bud	Sprout fresh weight (g)/total fresh weight (g)
Control	2.9 ± 0.6	1.4 ± 0.1	2.1 ± 0.2	8.5 ± 0.4
c6	9.2 ± 1.4	2.3 ± 0.3	4.5 ± 0.7	10.6 ± 0.8
c7	8.1 ± 0.7	1.7 ± 0.2	5.5 ± 0.5	11.6 ± 0.5
c9	23.5 ± 2.7	3.2 ± 0.3	7.9 ± 0.8	9.4 ± 0.4
c14	11.5 ± 2.8	2.0 ± 0.2	5.9 ± 1.0	10.9 ± 0.5
c15	8.7 ± 1.1	1.7 ± 0.3	6.3 ± 0.9	10.8 ± 0.6
c16	7.0 ± 0.94	1.4 ± 0.1	5.2 ± 0.6	11.3 ± 0.6
c18	8.6 ± 1.3	1.9 ± 0.3	5.0 ± 0.8	12.7 ± 0.9

Data were obtained from five independent plants clonally propagated from each individual transformant. Values are the mean ± standard error between independent potato tubers (n = 10–27). Tubers with 8–50 g fresh weight were harvested from non-senescing plants and stored for 6 months at 20°C in the dark.

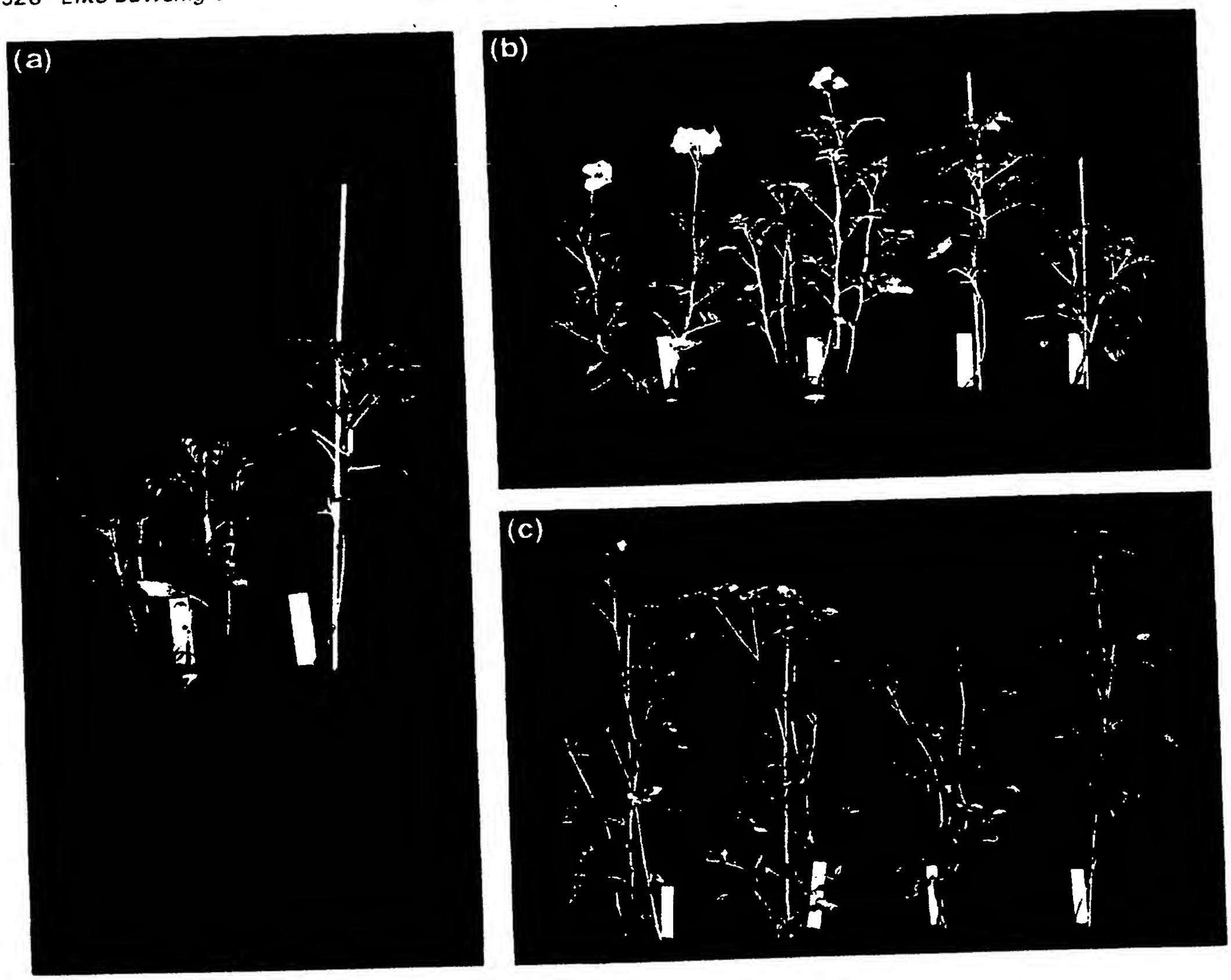

Figure 4. Altered sprouting of transgenic potato tubers leads to significant changes in further plant development.
(a) Changes in the number of stems formed per seed tuber. Potato plants with a strong reduction in cytosolic phosphorylase activity (c9, left) form more stems and produce a higher fresh weight of aerial parts than control plants (control, right).
(b) Changes in the onset of flowering. Transgenic plants (c16 and c9, left) show a premature flowering in comparison to control plants (control, right).
(c) Increase in the total flower set production. Inhibition of cytosolic phosphorylase frequently leads to a second or third inflorescence (c16-1 and c16-2, left), a phenomenon not observed in control plants (two controls, right).

cences. In contrast, wild-type plants flowered only once. The time to 50% flowering was six weeks after planting in some transgenic lines, compared with 12 weeks in untransformed control plants. More than 60% of the transformants investigated flowered twice, and about 20% even three times, with the same number of flowers in the first and second influorescences compared with wild-type plants but a reduced number of flowers in the third inflorescence. Upon harvest, not only was there an increase in the amount of above-ground biomass observed, but in addition the number of tubers, as well as the total tuber yield, had increased significantly (Table 3). Thus, in addition to leading to more shoots, alterations of behaviour with respect to both flowering and tuber yield were observed as a result of inhibiting the cytosolic phosphorylase.

Discussion

The reduction of the cytosolic isoform of phosphorylase has no pronounced effects on starch metabolism

The introduction of a chimeric gene containing the coding region for cytosolic phosphorylase in antisense orientation to the CaMV 35S promotor into potato plants results in a

Table 3. Influence on plant development of altered sprouting of transgenic potato tubers with decreased levels of cytosolic phosphorylase

Plant	Number of stems per plant	Total fresh weight of aerial parts (g) per plant	Number of tubers per plant	Total fresh weight of tubers (g) per plant
Control	2.2 ± 0.2	91 ± 4.5	5.6 ± 0.7	69.0 ± 4.1
c6	4.9 ± 0.5	126 ± 10.5	13.5 ± 1.4	95.0 ± 6.0
c7	6.6 ± 0.7	116 ± 6.5	10.8 ± 1.0	90.5 ± 4.3
c9	5.0 ± 0.3	124 ± 5.0	10.0 ± 0.7	92.5 ± 4.6
c14	4.5 ± 0.3	118 ± 3.9	9.6 ± 1.2	93.8 ± 4.4
c15	4.7 ± 0.4	124 ± 8.0	12.8 ± 2.5	94.5 ± 4.4
c16	4.5 ± 0.4	115 ± 7.5	8.8 ± 0.4	92.5 ± 2.8
c18	5.7 ± 0.4	128 ± 9.5	11.3 ± 0.7	82.5 ± 4.2

Data were obtained from independent plants clonally propagated from each individual transformant. Tubers with 8–50 g fresh weight were harvested from non-senescing plants. Values are the mean ± standard error between independent potato plants (n = 4–8; stem number per plant: n = 9–11). The results were confirmed in a second and third set of experiments (data not shown).

drastic reduction of the expression of Pho2-specific mRNA and the corresponding activity, in both leaves and tubers. Despite this reduction, no major change was observed in starch levels of leaves or tubers (at different developmental stages) of the transgenic plants compared with wild-type controls. When this result is taken together with the absence of observable alterations of starch structure and tuber formation (in plants generated from stem cuttings), these results strongly argue against a major involvement of the cytosolic isozyme in starch metabolism of potato plants.

The presence of α-1,4-glucan-metabolizing enzymes in subcellular compartments where no starch is synthesized is still not understood. It is conceivable that malto-oligosaccharides synthesized by cytosolic phosphorylase might penetrate the amyloplast membrane during starch synthesis thus providing primer units, or alternatively starch-derived malto-oligosaccharides could be degraded in the cytosol by the action of cytosolic phosphorylase. Amyloplast transport has been shown for glucose and maltose (Möhlmann *et al.*, 1995; Neuhaus *et al.*, 1995). However, evidence for transport of longer maltodextrins is still lacking. The fact that the HPAEC analysis did not reveal any changes in oligosaccharide levels and composition in crude tuber extracts from transgenic and wild-type plants could also be due to the fact that the analysis was performed with total extracts and no subcellular fractionation had been applied. Minor changes of specific oligosaccharides in one compartment could thus be masked by the presence of higher amounts of these oligosaccharides in a different compartment. Despite these possibilities, malto-oligosaccharides which are synthesized or degraded by Pho2 in the cytosol are unlikely to be a major intermediate in starch formation or degradation, as no changes in the amount and structure of starch were observed in the plants with lowered levels of Pho2 expression.

The decrease in reducing sugar content in sprouting tubers is probably not a direct effect of the lowered expression of the cytosolic phosphorylase

The only difference with respect to carbohydrate levels was measured in sprouting tubers. After keeping the tubers for 7 and 13 months in storage at 20°C, glucose and fructose levels were 50% lower in the tubers with reduced cytosolic phosphorylase levels compared with wild-type controls, whereas no differences were found with respect to starch and sucrose levels. The changes were not observed after shorter periods of storage, i.e. after time intervals during which the tubers can be regarded as dormant. As observed here, potato tubers generally accumulate sugars after prolonged periods of storage. This phenomenon is not necessarily linked to the emergence of sprouts, but rather to senescence, and widely regarded as 'senescent sweetening' (Burton *et al.*, 1992). Starch is degraded, and mainly sucrose but also the reducing sugars glucose and fructose accumulate. The lowered levels of the reducing sugars in the transgenic tubers do not affect the total soluble sugar content significantly in comparison with control tubers.

The reduction of glucose and fructose cannot be explained by the lowered levels of the cytosolic phosphorylase isozyme, as it is not involved in the interconversion of sucrose and the hexoses. This reduction most probably should be attributed to an invertase, because glucose and fructose accumulate, both in wild-type and transgenic tubers, in equimolar amounts.

The reduced levels of the hexoses cannot be explained on the basis of the data obtained. However, it is possible that this phenomenon is linked to the loss of apical dominance observed in these transgenic plants. A similar observation was made in a different experimental system, where apical dominance in potato tubers was broken by the removal of the emerging sprouts (Hughes and Fuller, 1984).

Inhibition of cytosolic phosphorylase influences sprouting and further plant development

Apical dominance in sprouting tubers, which can be exhibited by the youngest bud, is largely influenced by the physiological age of the seed tuber, and may be lost in later stages of development (Burton, 1989). A tuber is regarded as young when it is either dormant or if apical dominance is leading to the formation of only a single sprout. Ageing of tubers results initially in the formation of multiple sprouts, and, later on, in an increase of sprout branching. Senile tubers can show hair sprout growth or the direct formation of small tubers (Beukema and van der Zaag, 1990). In stored tubers of potato plants with reduced levels of cytosolic phosphorylase activity, apical dominance seems to be largely lost with respect to the sprouting process in comparison with wild-type plants, as formation of multiple and branched sprouts occurs almost immediately after dormancy is broken.

Tuber dormancy and sprouting capacity are postulated to be regulated by plant growth regulators such as gibberellins, abscisic acid, auxins and cytokinins. Gibberellins control sprout growth by breaking the dormancy of tubers (Bruinsma and Swart, 1970), while abscisic acid has a reverse effect (Blumenthal-Goldschmidt and Rappaport, 1965; Suttle, 1995). Both auxins and cytokinins can promote sprouting and apical dominance (Binns, 1994; Davies, 1987; Kumar and Knowles, 1993b). Although it cannot be excluded that the reduction of the cytosolic phosphorylase has some effect on the amount or activity of these growth regulators, there is no biochemical pathway suggesting a linkage between the phosphorylase and these hormones.

On the other hand, specific oligosaccharides are accepted as displaying biological activity and have been implicated in pathogen defence and plant growth, development and morphology (Albersheim *et al.*, 1983; Ryan, 1987). It is therefore tempting to speculate that in the transgenic plants discussed here, a modification of the structure of the heteroglycan which supposedly is the substrate for the cytosolic phosphorylase (Yang and Steup, 1990) has occurred, which in turn leads to the observed effects with respect to tuber sprouting.

The main problem in obtaining further evidence for the existence (natural occurrence) of oligosaccharins in tubers is the extremely low concentration at which oligosaccharides exhibit their biological activities and at which they are therefore likely to occur in living tissue (Fry, 1986). It was not possible to find any changes in oligosaccharide levels in the transgenic tubers. In order to address this question further, heterogeneous preparations of oligomers generated by enzymatic fragmentation of the cytosolic heteroglycan (purified from potato tuber) will be used in future bioactivity studies.

The altered sprouting behaviour of tubers with reduced levels of cytosolic phosphorylase is correlated with an increase in tuber yield per plant

The yield per potato plant is to a larger extent dependent on the amount of stems formed. The alteration in tuber sprouting of the transgenic plants leads to more stems, from which stolons can be initiated, and therefore to the production of more tubers. The harvest index (total fresh weight of tubers/total fresh weight of shoots and tubers) is not altered and the increase in yield is therefore only interpreted as a result of the changed sprouting behaviour. Whether this advantage can be exploited on the agronomical level has to be tested under field conditions. It is possible that potato seed tubers with reduced cytosolic phosphorylase activity could be used to achieve an optimal stem density with reduced amounts of seed potato material.

The reason for the difference in flowering, leading to a significant reduction in the time to flowering in the transgenic plants, is probably also related to the changed sprouting behaviour, as sprout emergence was observed significantly earlier in the transgenic plants than the controls.

In conclusion, the unaltered starch quantity and quality in tubers, and the effects we have observed in transgenic plants with reduced levels of cytosolic phosphorylase activity on sprout growth and flowering, indicate that cytosolic phosphorylase does not participate in starch metabolism, but rather fulfils other physiological functions in the plant cell that are connected to the regulation of plant growth and development. A detailed metabolic analysis of the transgenic plants, as well as a thorough study of their heteroglycan components, might help to elucidate the role of cytosolic phosphorylase in plants.

Experimental procedures

Plants, bacterial strains and media

Potato plants (*Solanum tuberosum* L. cv. Désirée) were obtained from Vereinigte Saatzuchten eG (Ebstorf, Germany). Plants in tissue culture were maintained under a 16 h light/8 h dark regime (irradiance 200 $\mu E\ m^{-2}\ sec^{-1}$) on MS medium (Murashige and Skoog, 1962) containing 2% sucrose. Plants for biochemical analysis were cultivated in the greenhouse in soil under a daily regime of 16 h light (250 $\mu E\ m^{-2}\ sec^{-1}$; 22°C) and 8 h dark (15°C). Each plant was grown in a pot (200 cm^2, 15 cm deep) and watered daily. Tubers were harvested four months after transfer from tissue culture to the greenhouse or after planting seed tubers in the greenhouse.

Escherichia coli strain XL1-Blue (Bullock *et al.*, 1987; Stratagene, Heidelberg, Germany) was cultivated at 37°C in YT-Medium (8 g l^{-1} Bacto tryptone, 8 g l^{-1} Bacto yeast extract, 5 g l^{-1} NaCl, pH 7.0) using standard techniques (Sambrook *et al.*, 1989). *Agrobacterium tumefaciens* strain C58C1 containing plasmid pGV2260 (Deblaere *et al.*, 1985) was cultivated in YEB medium (Vervliet *et al.*, 1975).

Reagents

Enzymes for DNA restriction and modification were obtained from Boehringer Mannheim (Ingelheim, Germany) and New England Biolabs (Danvers, MA, USA). Enzymes for carbohydrate determinations were obtained from Boehringer Mannheim. Radiochemicals and nylon membranes (Hybond N) were obtained from Amersham Buchler (Braunschweig, Germany). All other chemicals were obtained from Boehringer Mannheim, Sigma Chemical Co. (St Louis, MO, USA) or Merck (Darmstadt, Germany).

Recombinant DNA techniques and construction of a chimeric gene for antisense expression of cytosolic phosphorylase in transgenic potato plants

Standard procedures were used for recombinant DNA work (Sambrook *et al.*, 1989). For the construction of the cytosolic phosphorylase antisense gene, a 2 kb *Sma*I/*Asp*718 cDNA fragment was amplified from a λZAPII library of potato tuber (Kossmann *et al.*, 1991) using polymerase chain reaction technology (Perkin-Elmer, Norwalk, CT, USA). Synthetic oligonucleotides were synthesized by TibMolBiol (Berlin, Germany) based on the published sequence for potato cytosolic phosphorylase (Mori *et al.*, 1991). The sequence of the primers was designated such that the 3'end of the coding region for cytosolic phosphorylase cDNA was amplified and an additional *Asp*718 restriction enzyme recognition sequence at the 3'end of the amplified DNA was introduced: p5'-*Pho*2 GTCCCCGGGAAATTGTAAGGCATG and p3'-*Pho*2 TTAGGTACCAGGTTCA GAAGTGTGC. The amplified DNA was digested with *Sma*I and *Asp*718 and cloned into the *Asp*718/*Sma*I sites of pBluescript SK– giving rise to the plasmid pSK-Pho2. The fragment was introduced subsequently into the *Asp*718/*Sma*I sites of the binary plant expression vector pBinAR (Bevan, 1984; Höfgen and Willmitzer, 1990) between the 35S cauliflower mosaic virus (CaMV) promotor (Franck *et al.*, 1980) and the polyadenylation signal of the T-DNA octopine synthase gene (Gielen *et al.*, 1984), resulting in the plasmid p35SαPho2^{Kan} (see Figure 1). The antisense construct was used for direct transformation of the *Agrobacterium tumefaciens* strain C58C1:pGV2260 (Höfgen and Willmitzer, 1988).

Plant transformation

The construct p35SαPho2α^{Km} (Figure 1) was used to transform *Solanum tuberosum* L. cv. Désirée via *Agrobacterium tumefaciens* strain C58C1:pGV2260 as described by Rocha-Sosa *et al.* (1989).

Northern blot analysis

A collection of various independent transgenic lines was used for all experiments. Total RNA was extracted from frozen plant material according to Logemann *et al.* (1987). The RNA was denatured in 40% formamide, subjected to agarose gel electrophoresis (1.5% agarose, 15% formaldehyde; 20 µg RNA/lane) and blotted in 20% SSC onto nylon membranes (Hybond N, Amersham Buchler, Braunschweig, Germany). Membranes were hybridized at 42°C in buffers containing polyethylene glycol and formamide as described (Amasino, 1986). Radioactive labelling of DNA probes was performed using a multiprime labelling kit (Amersham Buchler). Filters were washed twice in 2×SSC, 0.1% SDS for 30 min at 60°C and once in 0.2×SSC, 0.1% SDS for 30 min at 60°C. The 2.1 kb long *Pst*I-insert of plasmid pSK-Pho2 was used for the labelling reaction. After the membranes had been washed they were exposed to X-Ray films (Kodak X-Omat AR, Siemens, Berlin, Germany) for autoradiography.

Native polyacrylamide gel electrophoresis

For affinity electrophoresis, a non-denaturing discontinuous system was used, essentially according to Steup (1990). Crude protein extracts were separated in anionic acrylamide gels containing 2.4% glycogen. Frozen tissues were homogenized in extraction buffer (100 mM HEPES–NaOH, pH 7.5, 1 mM EDTA, 10% (v/v) glycerol, 5 mM DTT, 7.1 µmol Na_2SO_3, 3.6 µmol $Na_2S_2O_5$) and the homogenate was clarified by centrifugation. Protein content was determined by the method of Bradford (1976) using BSA as a standard. Equal amounts of protein were applied to each lane (10–30 µg/lane). PAGE was performed at 4°C for 4 h at 100 V using a MiniProtean II apparatus from BioRad (Richmond, CA, USA). For phosphorylase activity staining following electrophoresis, the separation gel was equilibrated for 1 h in 100 mM citrate-NaOH (pH 6.0; 4°C; one change of buffer). It was then incubated overnight at 20°C or for 1 to 2 h at 37°C in a mixture containing 20 mM glucose-1-phosphate and 100 mM citrate-NaOH (pH 6.0). Iodine staining of the gel was performed with Lugol's solution for 5 min, and the gel destained by washing in water several times for extended periods under gentle shaking. Glucan synthesizing enzyme activities were visible as blue staining bands.

Carbohydrate analysis

Tuber slices or leaf discs (approximately 50 mg) were extracted with 80% (v/v) ethanol or with water at 80°C. The determination of starch, fructose, sucrose and starch was performed as described (Müller-Röber *et al.*, 1992).

The density of tubers was determined according to von Schéele *et al.* (1937).

For microscopic analysis of the size and morphology of starch granules an Axiophot photo microscope (Zeiss, Oberkochen, Germany) was used.

The amylose content of the starch was measured iodometrically according to Hovenkamp-Hermelink *et al.*, (1988).

The phosphate bound to the starch at the C6 position was determined as glucose-6-phosphate released after acid hydrolysis according to Nielsen *et al.* (1994).

Analysis of the side-chain distribution and the α- and β-amylolytic hydrolysis products of isolated starch was performed by dissolving 10 mg starch in 100 µl 0.1 M NaOH for 1 h at 95°C. After diluting the sample with 900 µl water, 150 µl 1 M sodium citrate (pH 5.0) was added and the starch was debranched with 300 units of isoamylase from *Pseudomonas amyloderamosa*, or hydrolysed with 300 units of α-amylase from *Bacillus amyloliquefaciens* or β-amylase from *Ipomea batatas* for 24 h at 37°C. A 100 µl aliquot of the hydrolysed samples was subjected to HPAE–PAD chromatography (Carbo PAC PA-100 column; Dionex, Idstein, Germany; flow 1 ml min^{-1}; buffer A: 150 mM NaOH; buffer B: 1 M sodium acetate in buffer A). The following gradient was applied: 0–5 min 100% A; 5–20 min 85% A, 15% B, 20–35 min 70% A, 30% B (linear); 35–80 min 50% A, 50% B (convex).

The paste behaviour of 8% (v/v) starch suspensions was investigated using a Rapid Visco Analyser (RVA; Newport Scientific Pty Ltd, Warriewood, Australia). The following temperature programme (13 min) was applied: 0–0.1 min at 50°C, 960 rpm; 0.1–1 min at 50°C, 160 rpm; 1–4.45 min heating to 95°C, 160 rpm;

4.45–7.15 min hold at 95°C, 160 rpm; 7.15–11 min cooling to 50°C, 160 rpm.

The strength of the gel which was obtained after incubating a RVA starch paste for 24 h at room temperature was tested using a Stable Micro Systems TA-XT2 Texture Analyser (Godalming, Surrey, UK).

Analysis of the content and distribution of malto- and other oligosaccharides in tubers was performed by subjecting water- and ethanol-soluble tuber extracts to HPAE-PAD chromatography, as described above, before and after digestion with amyloglucosidase from *Aspergillus niger* for 24 h at 37°C. The ethanol-soluble extracts were dried and dissolved in water prior to digestion and HPAEC-PAD analysis.

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